Expression of immune checkpoint molecules of T cell immunoglobulin and mucin protein 3/galectin-9 for NK cell suppression in human gastrointestinal stromal tumors

Hideo Komita1, Shigeo Koido1,2, Kazumi Hayashi2, Shin Kan2, Masaki Ito2, Yuko Kamata2, Masafumi Suzuki3 and Sadamu Homma2

1Division of Gastroenterology and Hepatology, Department of Internal Medicine, 2Division of Oncology, Research Center for Medical Science and 3Department of Pathology, Jikei University School of Medicine, Tokyo, Japan

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Abstract. Monoclonal antibody therapy for immune checkpoint blockade has achieved promising results for several types of malignant tumors. For the future treatment of gastrointestinal stromal tumors (GISTs) by immune checkpoint blockade, expression of immune checkpoint-related molecules that suppress antitumor immunity in GISTs was examined. Infiltration of immune cell types into 19 GIST tissues was analyzed by immunohistochemistry, and expression of T cell immunoglobulin and mucin protein 3 (Tim-3) and programmed cell death-1 (PD-1) in the infiltrated immune cells was examined by immunofluorescence microscopy. The expression status of galectin-9 in the GIST tumor cells was also determined by immunohistochemistry. All the GIST tissues showed CD8+ T cell infiltration and 8 showed CD56+ natural killer (NK) cell infiltration, and the numbers of infiltrated CD8+ T and NK cells were strongly correlated. However, these CD8+ T and NK cells were CD69-negative inactivated cells. Tim-3 was expressed in the infiltrated NK cells in 6/8 (75%) of the GIST tissues. Expression of galectin-9, a ligand of Tim-3, was observed in 13/19 (68.4%) GIST tissues and all of the GIST tissues with Tim-3+ NK cell infiltration showed positive galectin-9 expression. No PD-1 expression in the infiltrated NK cells and neither Tim-3 nor PD-1 expression was observed in the infiltrated CD8+ T cells. Interaction between Tim-3 in infiltrated NK cells and galectin-9 in tumor cells may be involved in an immune checkpoint mechanism for suppression of antitumor immunity in GISTs. Blockade of the Tim-3/galectin-9 pathway may become a new strategy for GIST treatment.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract derived from the interstitial cells of Cajal (1). GIST develops in the stomach most frequently, followed by the small intestine, colon and esophagus. Expression of receptor tyrosine kinase KIT seems to be closely associated with GIST development, and 75-90% of GISTs elicit functional mutations of the KIT gene at exon 9, 11, 13 and 17 (2-4). Although tyrosine kinase inhibitors (TKIs) such as imatinib mesylate or sunitinib malate have been applied for the treatment of inoperable GISTs, tolerance to such agents has often been induced and the antitumor effects by such agents on GISTs is restricted (5,6). Accordingly, new therapeutic modalities for GISTs which are based on new biological mechanisms are urgently required.

Studies indicate that GIST has immunogenic properties which attract the antitumor immune response. Infiltration of immune cells into GIST tissues has been shown as possible immune responses between the tumor and immune cells (7,8). It has also been reported that NK cells and the interferon-γ status of GIST patients predict prognosis (9), and imatinib mesylate induced NK cell activation and promoted antitumor immunity to GIST (10). Of note, GISTs express various types of tumor-associated antigens recognized by specific cytotoxic T lymphocytes (11-14). However, there is no evidence indicating that host antitumor immunity inhibits the development of GISTs and no therapeutic modalities which could suppress GISTs by activation of antitumor immunity have been developed.

Recently, treatment with immune checkpoint inhibitors has provided monumental progress in cancer treatment (15,16). Some melanoma patients showed marked responses to treatment with a monoclonal antibody to cytotoxic T lymphocyte antigen-4 (CTLA-4) (17). Marked tumor regression, durable
antitumor effect and no autoimmune-associated adverse events of the therapy indicated that inhibition of tumor-associated immune-suppression could become a potential therapeutic modality to suppress tumor development (18). Recent studies have shown that the neo-antigens generated by somatic mutations may become targets recognized by specific CTLs (19). Blockade between programmed cell death-1 (PD-1) on T cells and programmed cell death ligand-1 (PD-L1) on tumor cells by monoclonal antibody treatment also showed a significant antitumor effect against melanomas as well as lung and renal cancers (20,21). Combined treatment with anti-CTLA-4 and anti-PD-1 treatment of melanomas may show high therapeutic efficacy which has never been observed before and may decrease the toxicity of anti-CTAL-4 by decreasing its dose (18). Besides these molecules, T cell immunoglobulin and mucin protein 3 (Tim-3) on immune cells and galectin-9 on tumor cells play an important role in immune checkpoint mechanism in malignancies and the blockade of the Tim-3/galectin-9 pathway is expected for future immune checkpoint therapy against malignant tumors (22,23). Exciting results by immune-checkpoint blockade has opened a new era for cancer therapy, and patients with various malignant tumors other than melanomas may be able to receive the benefits in the near future.

Considering that GISTs have immunogenic properties, it is possible that antitumor immune activity of GISTs may be inhibited by immune checkpoint mechanisms. Although immune checkpoint blockade would be expected as a noted method for GIST treatment, no data concerning the immune checkpoint mechanism of GISTs have been reported. In the present study, we demonstrated that the Tim-3/galectin-9 pathway may be involved in the immune checkpoint mechanism of GISTs.

Materials and methods

Patient characteristics. Human GIST tumors investigated in the present study were obtained from 2006 to 2014 from 12 male and 7 female patients ranging from 40 to 82 years of age (median 60 years). All samples were primary GIST tissues of the stomach, whose diameters were >30 mm in 16 cases and from 30-100 mm in 3 cases. All of the GIST tissue samples were obtained by surgical procedure, and no distant metastatic lesions were noted. All the GIST tissues were structured with spindle or epithelialoid cells with positive c-kit expression. None of the GIST cases were treated with tyrosine kinase inhibitors. Analyses of the GIST tissues were approved by the institutional review board according to the guidelines of the Jikei University School of Medicine and the Jikei University Hospital (25-299, 7434).

Antibodies. The primary antibodies for immunohistochemistry were as follows: anti-human CD69 rabbit polyclonal antibody (pAb), anti-galectin-9 rabbit pAb and anti-programmed death-1 (PD-1) rabbit pAb (all from Abcam, Cambridge, UK), anti-Tim-3 rabbit pAb (BioVision, Milpitas, CA, USA), anti-human CD4 mouse monoclonal antibody (mAb), IF6, anti-human CD8 mouse mAb, C8/144B and anti-human CD56 mouse mAb, 1B6 (all from Nichirei Biosciences, Tokyo, Japan), anti-FOXP3 mouse mAb, 236A/E7 (Abcam) and anti-human CD68 mouse mAb, PG-M1 (Dako, Glostrup, Denmark).

Secondary antibodies for immunohistochemistry were anti-mouse immunoglobulin goat pAb conjugated with peroxidase in Max-PO(M) and anti-rabbit immunoglobulin goat pAb conjugated with alkaline phosphatase in Max-PO(R) (both from Nichirei Biosciences).

Secondary antibodies for immune-fluorescence microscopy were goat anti-mouse immunoglobulin conjugated with Alexa Fluor 488 and donkey anti-rabbit immunoglobulin conjugated with Alexa Fluor 594 (both from Thermo Fischer Scientific, Waltham, MA, USA).

Immunohistochemistry by immune-peroxidase or alkaline phosphatase method. All stainings of tissue sections were performed on 4-µm formalin-fixed and paraffin-embedded tumor sections. Tissue sections were de-waxed in xylene and re-hydrated through decreasing concentrations of ethanol. Before immunostaining with the primary antibody, the sections were washed with phosphate-buffered saline (PBS) and incubated with 10% bovine serum in PBS for 60 min. They were then washed extensively with PBS and incubated with each primary antibody at a dilution of 1:100 at room temperature for 1 h. The slides were washed three times with PBS and incubated with secondary antibodies at room temperature according to the manufacturer's instructions. Each slide was immersed in hematoxylin bath for 1 min and washed with water. Tissue sections were re-hydrated in ethanol and cleared in xylene and mounted with permanent mounting media.

Enumeration of the immune cells in the GIST tissues. Numbers of CD8+ T, CD4+ T and CD56+ NK cells, CD68+ macrophages and FOXP3+ regulatory T cells were counted in 10 randomly chosen x40 microscopic fields.

Immunofluorescence microscopy. Formalin-fixed and de-waxed GIST tissue sections were incubated with the primary antibodies at a dilution of 1:100-1:200 at 4°C overnight. After being washed, they were incubated with goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 and donkey anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Thermo Fischer Scientific) at room temperature for 1 h. Immunofluorescence images were detected with an immunofluorescence microscope (BZ-9000 All-in-One; Keyence, Tokyo, Japan).

Correlation analysis. Correlation analyses were performed using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

Infiltration of the immune cells into GIST tissues. CD8+ T cells were infiltrated into all of the GIST tissues at various degrees of infiltration (Fig. 1). CD4+ T cells were also infiltrated into all of the GIST tissues, but fewer than that in the CD8+ T cells. Significant infiltration of CD56+ NK cells was observed in 8 GIST tissues (Fig. 1). Several cases showed macrophage infiltration. The number of Foxp3+ cells was extremely low in all of the GIST tissues examined (Fig. 1).

Correlation of the infiltration between each immune cell type was examined. Infiltration of CD8+ T and NK cells was found to be strongly correlated (Fig. 2, correlation coefficient...
The correlation of infiltration between CD4+ T and NK cells was low. Although the infiltration of CD8+ T cells was correlated with that of CD4+ T cells, the correlation was lower than that between CD8+ T and NK cells (Fig. 2, correlation coefficient 0.2109).

Positive Tim-3 but negative PD-1 expression is noted in the infiltrated NK cells in the GIST tissues. The characteristics of the NK cells infiltrated into the GIST tissues were examined. No CD69-positive NK cells were found in all of the GIST tissues (Table I). Tim-3 was significantly expressed in the

<table>
<thead>
<tr>
<th>CD69</th>
<th>Tim-3</th>
<th>PD-1</th>
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<tr>
<td>CD56+</td>
<td>Positive</td>
<td>Negative</td>
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0.6923. The correlation of infiltration between CD4+ T and NK cells was low. Although the infiltration of CD8+ T cells was correlated with that of CD4+ T cells, the correlation was lower than that between CD8+ T and NK cells (Fig. 2, correlation coefficient 0.2109).
infiltrated NK cells in 6 out of 8 GIST tissues (Table I and Fig. 3), but no GIST tissue showed positive PD-1 staining in the infiltrated NK cells (Table I).

**Galectin-9 expression in the GIST tumor tissues and corresponding expression of Tim-3 in the infiltrated NK cells.** Expression of galectin-9 in the GIST tissues was classified according to 4 grades: -, +/-, + and ++ (Fig. 4). All of the GIST tissues with positive galectin-9 expression showed a cytoplasmic staining pattern. Thirteen out of 19 GIST tissues (68.4%) were significantly positive (+ to ++) for galectin-9 immunohistochemical staining (Table II). Six GIST tissues with Tim-3+ NK cell infiltration exhibited positive galectin-9 expression (Table III).

**Table II. Expression of galectin-9 in human GIST tissues of the stomach.**

<table>
<thead>
<tr>
<th>Galectin-9</th>
<th>Total</th>
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<td>-</td>
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<tr>
<td>+/-</td>
<td>4</td>
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<td>+</td>
<td>8</td>
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<tr>
<td>++</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
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**Table III. Expression of galectin-9 in GIST tumor cells and Tim-3 in infiltrated NK cells in 8 GIST tissues with significant NK cell infiltration.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Galectin-9</th>
<th>Tim-3</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>2</td>
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<td>15</td>
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**Negative Tim-3 or PD-1 expression in the infiltrated CD8+ T cells in the GIST tissues.** All of the CD8+ T cells in the 19 GIST tissues were negative for CD69 staining (Table IV and Fig. 5). Furthermore, none of the infiltrated CD8+ T cells in the GIST tissues showed positive staining for Tim-3 or PD-1 (Table IV).

**Discussion**

In the present study, we found that galectin-9 was expressed in 68.4% of the human GISTs. Galectins elicit β-galactoside...
binding capacity with the conserved carbohydrate recognition domains (CRDs). Fifteen mammalian galectins have been identified to date with three subtypes among which galectin-9 shows tandem-repeat with two CRDs joined by a linker peptide (24). The multi-faceted and occasionally conflicting roles of galectin-9 in tumor biology have been demonstrated, and tumor progression, tumor cell adhesion, metastasis and immune escape are the most likely involved (25). Galectin-9 expression has been shown as a prognostic marker of malignancies indicating that galectin-9-negative malignancies show frequent metastasis and those with high galectin-9 expression are associated with prolonged survival (26,27). In fact, GISTs are associated with a rare incidence of recurrence after surgical resection and infrequent distant metastases (1,4). It remains unclear whether this low aggressiveness of GISTs is associated with galectin-9 expression and whether GISTs showing distant metastasis lack galectin-9 expression. Another important biological implication of galectin-9 is its association with cell cycle control and induction of apoptosis (28,29). Galectin-9 induces the apoptosis of various types of blood cancer cells (30,31). Galectin-9 may contribute to the prevention of tumor metastasis by blocking adhesion to the endothelium and extracellular matrix (32).

Previous studies have found that GISTs express known tumor-associated antigens such as Wilms' tumor-1 (11-14), suggesting that GISTs are immunogenic tumors (7). However, in the present study, all of the CD8+ T and NK cells infiltrated into the GIST tissues were CD69-negative inactivated cells. In spite of the immune cell infiltration, no immune-related tumor cell destructions were observed in the GIST tissues. These results suggest that antitumor immunity is strongly suppressed in GIST tissues. Immune suppression in GIST tissues by a macrophage-mediated mechanism or others may be involved (33,34).

Galectin-9 induces inactivation and apoptosis of T cells via Tim-3 receptor signaling (35,36) and also suppresses NK cell function via Tim-3/galectin-9 interaction (37-40). On the contrary, a promoting effect of galectin-9 on NK cell-mediated antitumor activity was also reported (41). Tim-3/galectin-9 interaction seems to be complicated. The Tim-3 receptor has been implicated as a negative regulator of adaptive immune responses and has been linked to T-cell dysfunction in chronic viral infections or cancer (42) and the regulatory roles of Tim-3 on innate immune responses have also been shown (38). In the present study, we found that NK cells that infiltrated into the GIST tissues were inactive and frequently expressed Tim-3. Considering that galectin-9, a ligand of Tim-3, was frequently expressed in the GIST tumor cells and Tim-3 was expressed in the infiltrated NK cells corresponding to galectin-9 expression in the GIST tumor cells, it is quite likely that the functions of NK cells in the GIST tissues were suppressed by an immune checkpoint mechanism mediated by the Tim-3/galectin-9 pathway. This suppressive effect may be achieved through a complex mosaic of inhibitory or activating receptors probably depending on the signal intensity, and Tim-3 signaling acts as a suppressor for NK-cell response in GISTs (38,43).

CD8+ T cells that infiltrated into the GIST tissues were all CD69-negative inactivated cells and no CD8+ T cells expressed Tim-3 or PD-1. Unfortunately, expression of PD-L1,
a ligand of PD-1, in the GIST tissues was undefined, since the results of PD-L1 staining of the GIST tissues was quite different depending on the type of anti-PD-L1 antibody used for immunohistochemistry. Considering the absence of PD-1 expression in the infiltrated CD8+ T and CD56+ NK cells, the PD-1/PD-L1 pathway is not involved in the immune checkpoint mechanism in GISTs.

Although tumor-associated antigens recognized by specific CTLs were expressed in the GISTs, adaptive antitumor immunity was deceased. We performed primary cultures of resected GIST tissues in IL-2-containing medium in some cases and observed vigorously proliferating mononuclear cells migrating from the GIST tissues. They mainly consisted of CD56+ NK and CD8+ T cells, and HLA-A24 restricted WT1 tetramer-positive CD8+ T cells were present. These findings indicate that adaptive immunity targeting known tumor antigens may have been primed but suppressed in the GIST tissues. It has been stressed that innate immune responses including NK cell response are an important process for the induction of antitumor immune activity (44) and that activation of NK cell-mediated immune response is closely associated with the following activation of adaptive immunity (45,46). Suppression of NK cell activity may be correlated with the impaired CD8+ T cell-mediated adaptive antitumor immunity in GIST tissues. Finally, blockade of the immune checkpoint mediated by Tim-3/Galectin-9 interaction may potentially re-activate NK cell activity and may be beneficial for the treatment of advanced GISTs.

References


